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THE PRODUCTION OF APOSYMBIOTIC HYDRA BY THE PHOTODESTRUCTION OF GREEN HYDRA ZOOCHLORELLAE

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Research progress and continued interest in the hydra-algae symbiosis is, to a great extent, owed to the existence of aposymbiotic animals. Aposymbiotic hydra are hydra that normally harbor green, *Chlorella*-like algae in their digestive cells but through circumstances described below, have become algal-free. Aposymbiotic hydra survive and reproduce as well as green animals, providing they are fed regularly (Muscatine and Lenhoff, 1965), and serve as important control and experimental organisms in many experiments examining the physiology and morphology of the symbiosis. For instance, experiments regarding the nutritional basis of the symbiosis (Muscatine and Lenhoff, 1965), investigations demonstrating the recognition of potential symbionts hydra (Pardy and Muscatine, 1970, 1973), studies detailing the ultrastructural aspects of the animal-algal association (Pardy, unpublished), and energetic evaluation of the symbiosis (Stiven, 1965), have all been possible due to the availability of aposymbiotic hydra.

While the existence of free-living aposymbiotic hydra in nature is unreported, algal-free animals occasionally arise in laboratory cultures as a result of the chance occurrence of an algal-free zygote (Lenhoff, 1965). Such aposymbiotic zygotes mature and by asexual budding, produce clones of white animals that do not normally become symbiotized in laboratory cultures. These aposymbionts, however, can be reinfected artificially with symbiotic algae (Pardy and Muscatine, 1973) and thus retain the capacity for symbiosis.

The occurrence of algal-free zygotes, however, is at best an uncertain and capricious process. Moreover, the production of hydra by sexual processes gives rise to genetic recombinants which may not have the same physiological characteristics as the asexually reproducing parent clone. Moore and Campbell (1973) have shown that inbred hydra give rise to zygotes that exhibit high mortality and that some zygotes exhibit morphological and developmental aberrations. The latter condition was first described by Lenhoff (1965), who discovered a mutant strain of nonbudding hydra that was produced by sexual processes.

Objections to the use of aposymbiotic hydra derived from algal-free zygotes described above are partly overcome by the production of aposymbiotic animals in the laboratory by chemically treating adult, green hydra. By means of a technique first described by Whitney (1907), green hydra are maintained in 0.5% glycerine. After several days of exposure to glycerine, some animals become pale green to white. From some of the white animals, clones of aposymbiotic hydra can be reared by means of asexual budding. Using this method, investigators are able to prepare routinely algal-free animals that are genetically identical to their symbiotized progenitors. Glycerination has been used to produce aposymbiotic hydra

from the following strains of *Chlorohydra viridissima*: Carolina (Muscatine and Lenhoff, 1965), Burnett (Park, Greenblatt, Mattern and Merril, 1967) and from *C. viridissima* of unknown origin (Stiven, 1965). In addition, Epp and Lytle (1969) have prepared algae-free specimens of *Chlorohydra hadleyi* using glycerine.

Not all green hydra are amenable to glycerination. Unsuccessful attempts to produce aposymbiotic hydra have been reported for *C. viridissima* Burnett strain (Epp and Lytle, 1969) and Florida strain *C. viridissima* (Muscatine, 1974). Moreover, Muscatine (1974) reports resistance to glycerination by a strain of hydra designated European; and in unpublished experiments, I have been unable to produce aposymbients from *Hydra viridis*, Florida strain or an English strain of green hydra.

In this paper, a new method of aposymbiont formation is described, involving the apparent photodestruction of symbionts, that is successful in obtaining clones of algal-free hydra from strains refractory to glycerination.

Materials and Methods

Experimental animals

Laboratory populations of the green hydra species, *Hydra viridis* (Florida strain) and a larger strain of green hydra, designated the English strain, were reared and maintained in M solution according to the methods of Lenhoff and Brown (1970). The English animals, a gift of Dr. L. Muscatine, are easily distinguished from the Florida hydra by their larger size and characteristic nematocyst dimensions. Mature, budding English hydra average a relaxed length of about 3–3.5 mm *vs.* a length of 2–2.5 for the Florida animals under similar conditions. The tentacle stenoteles of the English strain average 8.7×10.4 microns *vs.* 7×9 microns for the stenoteles of the Florida strain. Length and nematocyst dimensions are from personal, unpublished observations.

The animals used in experiments were harvested from logarithmically growing populations fed daily on freshly hatched *Artemia* nauplii and maintained at $18 \pm 1°$ C in a photoperiod incubator under a 12 hour light/dark regime. Only mature animals possessing one to three buds were used.

Irradiation of animals with intense light

Animals to be exposed to high light intensity were placed in 70 ml plastic tissue culture bottles filled with M solution. Each vessel was completely submerged in a transparent bath maintained at $15 \pm 1°$ C and continuously illuminated by 150 watt G. E. reflector flood lamps. The amount of radiation impinging on the culture bottles was controlled by varying the distance of the lamp above the experimental cultures. The amount of light energy reaching the surface of the culture bottles was measured using a Yellow Springs Model 65A radiometer. A spectral analysis of the lamps used as light sources was performed using an ISCO spectroradiometer. During some irradiation experiments, green hydra were simultaneously exposed to 10^{-6} M DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea, from K and K Laboratories, Inc., Plainview, New York]. DCMU is a specific photosynthetic inhibitor (Losada and Arnon, 1963) which interferes with the oxygen evolving photochemical system of chloroplasts.

In some experiments, animals were exposed to light that had passed through Baush and Lomb medium band-pass filters (numbers 90-4-460, 90-4-450, 90-4-620).

Examination of animals for the presence of symbiotic algae

Hydra were analyzed for the presence of algal symbionts by direct examination of animals under a 30× dissecting microscope. Animals exhibiting no trace of green color in any part of their bodies (column, tentacles, peduncle, buds) when examined against a white background were scored as "bleached." Alternatively, direct examination of digestive cells for the presence of algae was done by macerating whole animals (David, 1973). Maceration of intact animals were accomplished by placing hydra in 0.2–0.5 ml of a solution consisting of glycerine, glacial acetic acid and water (1:1:13, V/V). After 15–30 minutes the preparation was gently shaken, a drop of 15% formalin added and wet slides prepared of the resulting cells. Slides were examined using phase optics.

Spectrophotometric analysis

Green and aposymbiotic hydra tissues were assayed for the presence of pigments by extraction of 10–20 whole animals in 2 ml of absolute ethanol. The animals were extracted at 4° C for 24 hours, after which the methanolic extracts were scanned in the visible range (400–700 nm) using either a Beckman Acta II or Cary 14 dual beam scanning spectrophotometer.

Experimental cross infection of algal symbionts

In some experiments, English aposymbiotic hydra infected with Florida symbionts and Florida aposymbiotic animals infected with symbionts originating from English green animals were used. The infection of aposymbiotic animals is a simple procedure involving homogenation of the animal donors in M solution followed by the separation and purification of the algae via low speed centrifugation of host homogenate and repeated washing of the algae cells.

Dense slurries of symbionts are injected directly into the recipient hosts enteron by means of a glass micropipette. Following injection, the animals are maintained under normal culture (ambient light, daily feeding) conditions and are considered to be repopulated when they are green through the body column and tentacles—a process that takes about 10–15 days. Specific details of algal injection and aposymbiont repopulation are given in Pardy and Muscatine (1973).

Results

Green Florida and English strain hydra were initially exposed to light of 620 watts/m^2. After five days of continuous exposure, the animals were analyzed for the presence of green color as described earlier. Table I shows that nearly half (49.5%) of the Florida animals appeared bleached whereas none of the English animals became white.

I have used DCMU before in unpublished work in an attempt to rid English and Florida hydra of this algal symbionts but without success. In the present work another attempt was made, only this time coupling the exposure of the animals to

the inhibitor while simultaneously irradiating the animals at 620 watts/m². Under these conditions (with DCMU) almost all (99.3%) of the Florida hydra and a third (32.8%) of the English hydra appeared bleached (Table I).

As the English animals did not visibly bleach at 620 watts/m² in the absence of DCMU, in another experiment these animals and Florida strain hydra were subjected continuously to 1900 watts/m². Under these conditions all Florida animals were bleached at 72 hours at which time 62.5% of the English hydra were also bleached (Table I). Attempts to increase the irradiance further or to prolong exposure beyond 72 hours at 1900 watts/m², resulted in the disintegration of many of the animals in both strains.

To determine if the observed decrease in green color (=bleached) was due to a loss of symbionts and to evaluate the condition of the algae, samples of digestive cells from hydra were examined prior to irradiation and from animals judged bleached after five and seven days of continuous exposure to 620 watts/m² with or without DCMU. Table II shows the results of this experiment, and it is evident that the observed bleaching of the Florida strain hydra (with or without DCMU) and the English strain with DCMU results from a precipitous drop in the number of algal symbionts over the first five days. Algae continued to be lost from digestive cells in the 5–7 day interval with an increase in the number of cells lacking symbionts. Loss of algae from the Florida and English strains of hydra was most pronounced in those animals treated with DCMU (Table II) with all cells being void of symbionts in Florida strain (*vs.* 72% without algae in the absence of DCMU) and 95% of the digestive cells with no symbionts in the English strain (*vs.* 0% without algae in the absence of DCMU).

Within the digestive cells, the algal symbionts exposed to light at 620 watts/m² with or without DCMU exhibited a characteristic appearance. Compared with untreated controls, symbionts remaining in bleached animals appeared to be brownish in color and to be internally disrupted. Moreover the algae were located at the distal ends of the host's digestive cells sequestered in a large apical vacuole.

The results described above show that light and light DCMU are effective in causing the visual bleaching of green hydra. Moreover, microscopical analysis has shown that the observed loss of green color is due to elimination of the algal symbionts from the host's digestive cells. To see how complete and persistent the bleaching treatments were, 36 bleached animals of both strains were maintained under ambient light conditions and fed every three days. After three weeks the cultures were assayed for the presence of bleached or algal-containing hydra. The results of this experiment are shown in Table III. Almost all of the bleached Florida hydra (98.5%) remained algal free (100% in DCMU) and gave rise to aposymbiotic offspring by means of asexual budding. The bleached animals produced with or without DCMU showed no observable ill effects. The bleached English animals showed a lower proportion of permanently bleached animals (72.0%) indicating that the bleaching process had probably not been complete and that viable symbionts remained to reestablish the algal population. From both populations (Florida and English) clones of aposymbiotic hydra have been reared, the algal-free progney of which exceeds several thousands.

While both strains of hydra became bleached at 1900 watts/m², only the Florida strain produced aposymbiotic animals at 620 watts/m². To determine if the in-

TABLE I

The effect of various treatments on the "bleaching" of green hydra.

Hydra strain and experimental conditions	Number of animals bleached	Number of animals not bleached	Per cent bleached
Florida			
5 days, 620 watts/m²	76	77	49.5
5 days, 620 watts/m² + DCMU	128	1	99.3
72 hours, 1900 watts/m²	24	0	100.0
Florida			
3 days, 703 watts/m² 460 nm band-pass filter	0	24	0
3 days, 741 watts/m² 540 nm band-pass filter	0	24	0
3 days, 741 watts/m² 620 nm band-pass filter	15	9	62.5
English			
5 days, 620 watts/m²	0	148	0
5 days, 620 watts/m² + DCMU	45	92	32.8
72 hours, 1900 watts/m²	15	9	62.5
Florida hosts containing English symbionts— 5 days, 620 watts/m²	42	58	42.0
English hosts containing Florida symbionts— 5 days, 620 watts/m²	0	203	0

ability to bleach English animals at the lower irradiance was a function of symbiont resistance or host factors, English aposymbiotic recipients were cross infected with algae from Florida donors and likewise were infected with algae from English donors. After repopulation of the recipient hosts was complete, the animals were exposed to continuous irradiance of 620 watts/m². Table I shows that Florida hydra containing algae from English donors become bleached and the effectiveness of the treatment (42.0%) approaches that exhibited by the Florida animals when symbiotized by their native algae (49.5%). As before (Table I) English hydra, even though infected with algae from the Florida strain, did not become bleached, nor were any bleached individuals observed in this culture after 15 days of continuous exposure to irradiance of 620 watts/m².

To further explore the role of light in the bleaching phenomenon, Florida strain green hydra were exposed to light passed through medium band-pass filters as described in Materials and Methods. In each of the three experiments, the light source was adjusted to deliver a comparable amount of energy, though in one experiment involving a filter with a half-band width of 460 nm, heat problems necessitated an irradiance (703 watts/m²) of approximately 6% less than that used with the other filters (741 watts/m²). This small difference was considered insignificant. The high radiant energies required to bleach English hydra made it impractical to perform this series of experiments on these animals. Table II shows the results of these experiments and indicates that light composed mainly of the longer (red) wavelengths is most effective in causing bleaching of Florida hydra.

TABLE II

*Number of algal symbionts per digestive cell and digestive cells with no algae in Florida and European strains of hydra following five and seven days continuous exposure to light of 620 watts/m² with or without DCMU.**

Hydra strain	Day 0	Day 5		Day 7	
	Average number algae per digestive cell	Average number algae per digestive cell	Per cent of cells with no algae	Average number algae per digestive cell	Per cent of cells with no algae
Florida	15.98 ± 6.17	1.15 ± 2.30	67	1.09 ± 1.93	72
English	20.34 ± 6.38	19.42 ± 7.90	0	17.50 ± 7.15	0
Florida + DCMU	15.98 ± 6.17	0.07 ± 0.90	96	0	100
English + DCMU	20.34 ± 6.38	1.32 ± 2.52	69	0.21 ± 0.95	95

* Digestive cells were prepared by macerating 10 hydra together and counting the algae in 100 randomly selected digestive cells. Data expressed as mean ± standard deviation.

The spectral characteristics of the filters and the illumination source used in all experiments are shown in Figure 1A and B. Figure 1B shows that the unfiltered source yielded light predominately in the region passed by the red-band filter (550–650).

Spectrophotometric analysis of methanolic extracts of Florida and English green hydra revealed two distinct regions of light absorption: 400–500 nm and 580–680 nm. The absorption spectra for the two strains were identical and the curve for the Florida strain is shown in Figure 1C. Aposymbionts exhibited one peak at approximately 475 nm.

There existed the possibility that the epidermal cells of the green hydra act as light filters and that their efficiency as light screens would be a function of their thickness. Thus (as described earlier) 25–30 hydra of each strain were macerated and the cell thickness (length) of 50 randomly selected epidermal cells was measured using an eyepiece micrometer and phase optics. From these measurements, it could be determined that the English hydra had epidermal cells averaging 37 ± s.d. 6 μ thick, compared to the Florida animals which had cells averaging 25 ± s.d. 7 μ thick.

DISCUSSION

The results of the experiments described in this paper show that the symbiotic algae in two strains of green hydra (Florida and English) may be eliminated by exposing the hosts to intense light. I have called this phenomenon "bleaching." Tables I and II show that with time the animals exposed to strong light appear pale and that their bleached condition is due to the elimination of symbiotic algae from the host's digestive cells. Many of the bleached animals remain algal-free (Table III) and give rise to clones of aposymbiotic hydra. These aposymbionts are still receptive to algal symbionts as they can be reinfected with algae harvested from green hydra. Of interest is the fact that algae from different strains of hydra can be interchanged between different hosts. In the present work, English symbionts were successfully transferred to Florida hosts and *vice versa*. Apparently, both hydra strains "recognize" potential algal symbionts. Recognition, uptake, and rejection of algae by symbiotic hydra have been previously investigated (Pardy and

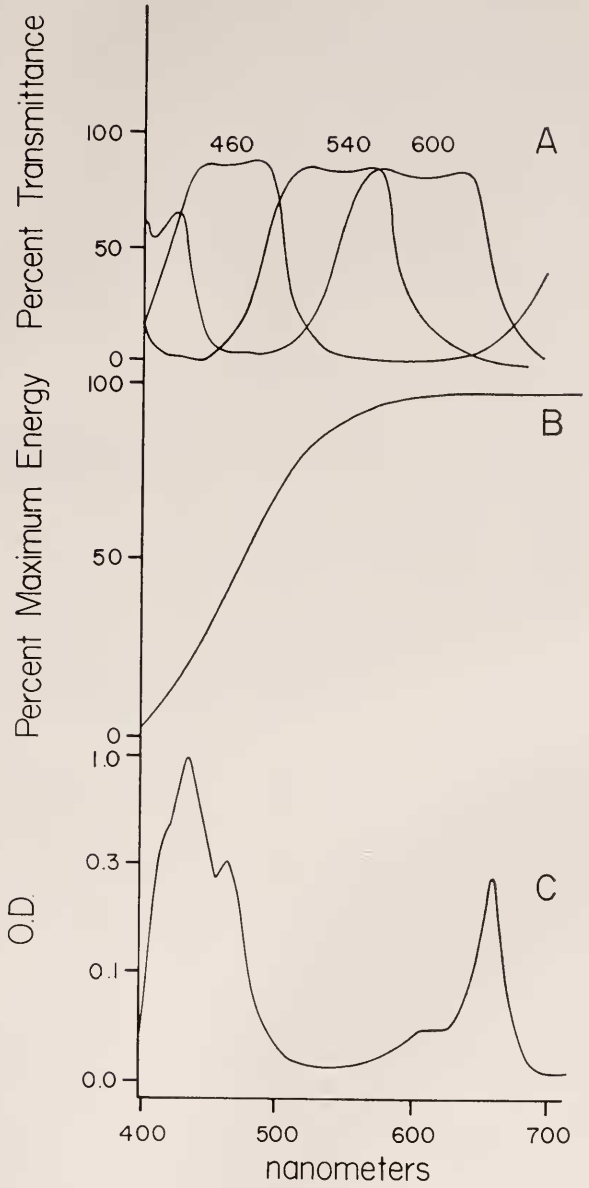

FIGURE 1. Curves of spectral absorbance of three medium band-pass filters used in experiments (A), spectral energy of light used in bleaching experiments (B), and optical density of a methanolic extract of green hydra (Florida strain) (C), plotted as a function of wavelength. The curves in (A) are reprinted from technical data sheets supplied by Baush and Lomb with permission.

Table III

Success of various bleaching treatments as measured by the number of bleached and green animals in a population of hydra three weeks post treatment.

Hydra strain and experimental treatment	Number of bleached hydra	Number of green hydra	Per cent remaining bleached
Florida			
5 days, 620 watts/m^2	67	1	98.5
5 days, 620 watts/m^2 + DCMU	66	0	100.0
English			
5 days, 620 watts/m^2 + DCMU	140	45	72.0

Muscatine, 1973). In this work (Pardy and Muscatine, 1973), it was shown that hydra can recognize potential symbionts and reject non-symbiotic algae. It is possible that the symbionts resident in the Florida and English strains of green hydra are identical; however, in a study recently completed (Pardy, 1976), it was found that the Florida and English symbionts may be distinguished on the basis of their ultrastructure.

From Table I it is evident that the English strain of hydra (or their algae) is less sensitive to the effects of intense light than the Florida animals in 620 watts/m^2 but is bleached when the irradiation is increased (Table I). Evidence derived from the cross transfer of algae between the two host strains suggests that the apparent resistance of the English algae to the effects of irradiance at 620 watts/m^2 is a function of the host in which it is resident (Table III). When algae are in Florida hosts, they are subject to the effects of light at 620 watts/m^2 while Florida algae in English hosts appear to have gained immunity. The bleaching of European hosts that takes place at 1900 watts/m^2 suggests that the protective mechanism existing in these animals may be overridden. While the nature of the protective mechanism is not known exactly, it may be related to the larger cell size evidenced by the European animals. To reach the endosymbiotic algae, light in the external environment must traverse the epidermal epithelium which may act as a light screen. The thickness of these cells, which in the European hosts is 1.3 times that of the Florida animals, may be the basis for the failure of the English animals to bleach at the lower irradiance.

The role of high intensity light in causing elimination of algal symbionts is not thoroughly understood, though there are at least two possible explanations. Unfavorable conditions such as elevated temperature and starvation have been shown to cause corals and sea anemones to rid themselves of symbionts (Muscatine, 1974). Likewise, intense light might act directly on the animal tissue inducing some unfavorable physiological condition causing the host to expel its symbionts. That high intensity light may adversely affect the animals was indicated by the disintegration of hydranths (Florida and English) when exposed to 1900 watts/m^2 for periods exceeding 72 hours. At this high intensity, however, the death and subsequent release of metabolites by a greater number of moribund algae cannot be completely ruled out as a factor in causing the demise of the hydra.

Alternatively intense light could act directly on the algal symbionts to cause

the destruction of photosynthetic pigments or other components of the photosynthetic apparatus. When chlorophyll pigment in algae becomes oversaturated with light quanta, it is degraded and the enzymatic systems associated with carbon dioxide fixation are inactivated (Steemann-Nielsen, 1962). The photodestruction of chlorophyll, however, takes place predominantly in the blue region of the spectrum (Soeder and Stengel, 1974) which is ineffective (Table I) in causing the bleaching of green hydra. The experiments shown in Table I and the measurements depicted in Figure 1 clearly implicate light in the red portion of the spectrum as being the active principle in the bleaching phenomenon. Absorption measurements on green hydra extracts reveal peaks (Fig. 1C) that are characteristic of green algae chlorophylls which absorb maximally in two regions—400–500 nm and 600–700 nm (Bogard, 1962).

Thus, while the chlorophyll pigments may not be undergoing photodestruction, it is possible that some red absorbing component associated with photosynthesis is being degraded. Further support for this argument follows from the observation that DCMU hastens the bleaching of Florida hydra and is essential for the elimination of algae from English hydra at 620 watts/m^2. DCMU, a specific photosynthetic inhibitor, has been shown to cause the bleaching of chlorophyll pigments as well as inhibit oxygen evolution and $^{14}CO_2$ incorporation (Zweig, Hitt and Cho, 1969). Recently Pardy and Dieckman (1975) have shown that DCMU inhibits photosynthesis of symbiotic algae *in situ*. It must be added that in unpublished experiments, I have been unable to cause bleaching with DCMU at ambient levels of light usually employed during culture of green hydra.

Finally, the disrupted appearance of the symbionts, when viewed under the microscope tends to suggest that intense light acts on the algae directly. These degraded algae are in striking contrast to the otherwise normal appearing host digestive cells within which they are contained.

Following photodestruction, the algal symbionts are removed from the host's digestive cells probably by emiocytosis. Once in the coelenteron, the algae are swept out *via* the water currents associated with respiration and elimination. In previous work (Pardy and Muscatine, 1973) it was shown that heat-inactivated symbionts were expelled from digestive cells following their localization in a large apical vacuole. Viable symbionts normally reside in individual vacuoles (Oshman, 1967) located at the base of the digestive cell. The expulsion of algae following exposure to high intensity light appears to be a process similar to the removal of heat-inactivated algae. How the host cell recognizes dead or moribund cells and moves them from the base of the cell to the apex for elimination is unknown. In a review, Muscatine (1974) cites his unpublished observations on algae in hydra treated with glycerine. According to Muscatine (1974), glycerine brings about the degradation of symbiotic algae followed by their elimination from the host. Hence the removal of pathologic symbionts appears to be a generalized response in green hydra, though the mechanism is unknown.

It is now possible to prepare aposymbiotic green hydra from strains refractory to glycerination. Moreover I have been able to produce aposymbionts of the English strain—a strain from which the existence of aposymbionts has yet to be reported. The importance of these algal-free English animals cannot be overstressed as these hydra (green and aposymbiotic) differ morphologically from the other

strains as described earlier (see also Muscatine, 1974) and, from unpublished observations, exhibit a variety of physiological characteristics (growth rate, metabolic rate, phototaxis) different from other green hydra.

With the advent of aposymbiotic forms of this strain together with the ability to cross-infect it with algae from other strains, new research avenues into the hydra-algae symbiosis are possible. For instance, in a recent work (Pardy, 1976), the ultrastructure of the algal symbionts residing in the English hydra were found to differ from that of the Florida symbionts. By making reciprocal algal crosses into aposymbionts prepared by bleaching, it could be seen that the host strain may determine the morphology exhibited by the algal symbionts. Such studies were made possible by the ability to produce English aposymbionts.

To the list of methods which give rise to aposymbiotic hydra from green animals (algal-free zygotes, glycerination) can now be added the photodestruction of symbiotic algae. How widely applicable this technique is to other strains of hydra or to other symbiotic species is not known although its use is presently being investigated on symbiotic protozoa and sea anemones.

Summary

1. Florida strain, but not the English strain of green hydra, are bleached by light at 620 watts/m^2.

2. English strain animals are bleached at 620 watts/m^2 in the presence of DCMU (a photosynthetic inhibitor) which also increases the bleaching effect in Florida hydra. English animals are also bleached by irradiation with 1900 watts/m^2.

3. Aposymbiotic clones that remain algal-free may be grown from bleached animals of both Florida and English strains.

4. Florida strain hydra containing English algae are bleached at 620 watts/m^2 but English hydra containing Florida algae are not.

5. The bleaching of Florida hydra takes place with light occurring in red region of the visible spectrum and probably involves the photodestruction of the photosynthetic system of the algal symbionts.

6. The bleaching of green hydra ultimately results from the removal of symbionts from the host's digestive cells.

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